Role of *Azotobacter* sp. On Nitrogen Uptake and Growth of Soybean (*Glycine max* (L.)Merrill) on Saline Soil

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Abstract--Biofertilizationby using *Azotobacter*for soybean on saline soils is needed to solve the problem of low soil fertility, especially in providing macronutrient nitrogen. The objective of this study was to determine the influence of *Azotobacter*sp. inoculation on nitrogen uptake and vegetative growth of soybean (*Glycine max* (L.) Merrill) in saline soil as well as on viability of *Azotobacter* sp. in rhizosphere and available nitrogen in saline soils. The potexperiment has been set up inrandomized block design with seven treatments and four replicate each. The treatmentswas soil inoculation with different isolate and concentration of liquid culture of *Azotobacter* sp. as follows *Azotobater*K4 0.5%,*Azotobater* K4 1.0%, *Azotobater*S2 0.5%, *Azotobater*S2 1.0%, K4+S2 0.5% and K4+S2 1.0%.The results showed that inoculation of *Azotobacter* sp. potentially increased N uptake,plant height, number of leaves, and shoot-root ratio of soybean grown in saline soil. Soilinoculationwith*Azotobacter*sp. alsoincreasedavailable N in soil andpopulation of *Azotobacter* sp., in soybean rhizosphere.

Keywords: *Azotobacter* sp., saline soil, soybean, nitrogen

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INTRODUCTION

The utilization of the Indonesian coastal zone in agriculture is still low because of the salt-affected soil problem (saline soils).Saline soilsare characterized by acidity ofless than 8.5, maximal exchangable sodium percentage (ESP) of 15%, and maximal sodium adsorption ratio (SAR) of 13 (Tan, 1995). Electrical conductivity(EC) of saline soil is more than 4 dS m^{-1} at 25^{o}C, and generates an osmotic pressure of about 0.2 Mpa which inhibit plant growth (Dhanraj *et al.*, 2012; Munns and tester, 2008).

Nitrogen (N) availability in saline is limited and hence inhibitssynthesis ofamino acids, enzymes, and chlorophyll, which in turn disturb the growth and metabolic processes of plants (Gomes *et al.*, 2014). Fertility of saline soils can be improved by using biofertilizer which fix dinitrogen (N_2). *Azotobacter*sp. is a non-symbiotic nitogen-fixing soil bacteria widely used as biofertilizer which abletochange dinitrogen (N_2) to ammonium (NH_4^+) at the rate of of 20 kg N/ha/per year (Kizilkaya, 2009). *Azotobacter* sp. is asPlant Growth Promoting Rhizobacteria(PGPR)dominantly found in agricultural soil and rhizosphere of important crops. The bacteria enableto solubilize inorganic phosphate, and produce phytohormone auxin, gibberellins, and cytokinins (Hindersah and Simarmata, 2004; Vikhe,2014).According to Omer *et al.* (2016)*Azotobacter*sp. wasreported elsewhere toproduce exopolysaccharide (EPS) in saline condition which can reduce the content of Na^+ in plants, so that more N can be uptake by plants.

Previous studies have shown that*Azotobacter* sp. inoculationhas positive effect on plant growth and production of soybean, an important protein source in Indonesia. However, effectiveness of *Azotobacter*sp. isolates on soybean grown on saline soils have not been widely reported.Application of *Azotobacter*sp. on saline soils by using indigenous isolates will optimize their adaptability and activity. Salt tolerance characteristics of *Azotobacter*sp. has been reported. Inoculation of *Azotobacter vinelandii*on maize grown in soil with EC up to 20 dS m^{-1},still increased length and weight of roots (Naz *et al.*, 2012).Soleimanzadeh *et al.*(2010)reported thatinoculation of *Azotobacter* liquid culture with cell density of10^8 CFU mL^{-1}on saline soils reduced the use of inorganic nitrogen fertilizer up to50% and increased growth of*Helianthus annuus* L.The study of *Azotobacter*sp. inoculation in soybean on saline

soils is important to enhance soybean production in Indonesia especially for soybean var. Grobogan which showed salt tolerance up to 9 dS m^{-1}(Triyani *et al.,* 2013).The objective of this pot experiment was to determine the role of two isolate of*Azotobacter*sp. to increase growth of soybean and their N uptake in saline soils; and to study the effect of *Azotobacter*sp. inoculation onpopulation of*Azotobacter* sp. in the soybean's rhizosphere and N availability in soil.

MATERIALS AND METHODS

The experiment was conducted from August 2016 until October 2016 at Ciparanje experimental field belong to Faculty of Agriculture, Universitas Padjadjaran, Jatinangor, West Java, Indonesia. The altitude of this area is 750 m above sea level.

Azotobacter sp. isolates K4 and S2has been isolated from saline soil in Karawang and Subang, Indonesia with EC of 5.37 dS m^{-1} and 4.74 dS m^{-1}respectively; and were cultured in Ashby's nitrogen-free media. Saline soilsfor pot experiment has been collected from paddy field atSubang, West Java, Indonesia.Soybean grown in this pot experiment was local soybean variety known as var.Grobogan having maximal yield of 2,77 t/ha.

The soil contain79% of clay withEC of 5.95 dS m^{-1};pH of 6.75 (neutral); organic-C of 0.65%; N-total of 0.21%; P and K potentialwere98.67 and 72.55 mg 100 g^{-1} respectively. Cationexchange capacity of soil was 13.52 cmol kg^{-1}; andits base saturation was 98.96%.One kg of soil was mixed with cow manure at the rate of 20 t ha^{-1} in black polyethylene bag (polybag) and was incubated for 7 days before planting. Decis 25 EC insecticide was sprayed wheninsect attacked more than 10% of experimental plant.

Experimental Set Up

This experiment tested seven liquid culture *Azotobacter*sp. inoculation treatments:
A: without inoculation (control);
B: 0.5% (v/v) of *Azotobacter* sp. isolateK4
C: 1.0% (v/v) of *Azotobacter* sp. isolateK4
D: 0.5% (v/v) of *Azotobacter* sp. isolate S2
E: 1.0% (v/v) of *Azotobacter* sp. isolateS2
F: 0.5% (v/v) of *Azotobacter* sp. isolateK4+S2
G: 1.0% (v/v) of *Azotobacter* sp. isolateK4+S2.
Each treatment was repeated four times.

Pure liquid culture of *Azotobacter* has been prepared by adding5 mLof physiological NaCl to one slant of *Azotobacter*sp. before bacterial colonies from the slant surface was mixed with liquid and poured into 100 mL of liquid Ashby's medium. The culture was incubated for three days for 72 hours atroom temperature of 24-26^{o}Con gyratory shaker of 115 rpm. Cell counts of *Azotobacter* sp. in liquid culture whichmeasured using a haemocytometer soon at last day incubation was attained 10^{8}cfu/mL. Mixed culture of isolate K4 and S2 has been prepared by mixing up both liquidinoculant(1:1).

Liquid inoculants of *Azotobacter* sp. of 10 mL werediluted in 50 mL unsterilized aquadest and sprayed on the soil in polybagexcept the control treatment, then incubated three days. Two seeds of soybean were sown in each polybag and maintained until the end of the vegetative phase at 35 days after sowing (DAS). Plant height, number of leavesof soybean was measured at 28 and 35 days. The shoot and root biomass was heated at 60^{o}Cand weighed to obtain shoot-root ratio. Available nitrogen (N-NH_4^+ and N-NO_3^-), N uptake, and population of total *Azotobacter*in the rhizosphere has been analyzed at the end of experiment.

RESULTS AND DISCUSSION

Available Nitrogen (N-NH_4^+ and N-NO_3^-) andN Uptakeof Soybean

Table 1 showed that available N (N-NH_4^+ and N-NO_3^-) and N uptake were increased after inoculation of *Azotobacter* sp.The highest available N (0.022 ± 0.010%)was obtained in inoculation of *Azotobacter* sp. K4+S2 1.0%. Higher N uptake (1.68 ± 1.086g plant^{-1}) was showed by plant inoculated with *Azotobacter* sp. K4+S2 of 0.5%. Inoculation of 1,0% of mixed *Azotobacter* sp. inoculant enhanced N-NH_4^+ and N-NO_3^-up to 45.45% and 37.78% respectively compared to control. *Azotobacter* sp. K4+S2 inoculation with a concentration of 0.5% also increased the N uptake up to 60.12% compared to control.

*Azotobacter*has the ability to produce phytohormones like auxin, gibberellin, and cytokinin which stimulates root growth to increase the ability to absorb N from the soil (Hindersah and Simarmata, 2004).In this experiment, the addition of cow manure (33.53% of organic-C) also involved in the improvement of available nitrogen since it provides nutrients for plants and increase organic matter content on saline soils as carbon source for *Azotobacter* sp. to proliferate and fix nitrogen (Jnawali *et al.*, 2015).

Table 1. The effect of*Azotobacter* sp. inoculation on N-$NH_4^+$and N-NO_3^- as well as N uptake on soybean shoot.

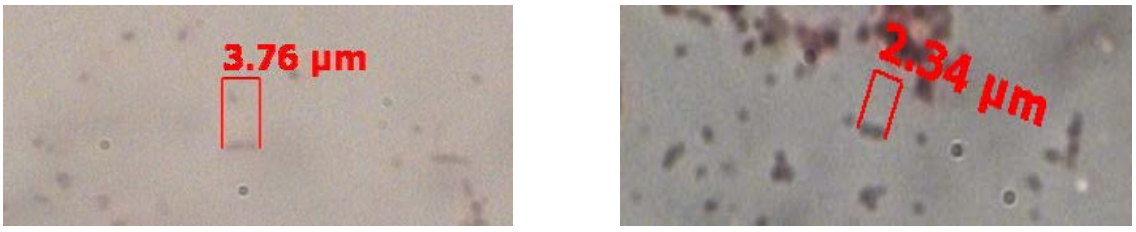
Treatments	Available N (%)		N uptake (g plant^{-1})
	NH_4^+	NO_3^-	
A = Without inoculation	0.012 ± 0.007	0.056 ± 0.014	0.67 ± 0.227
B = *Azotobacter* sp. K4 0.5%	0.015 ± 0.006	0.065 ± 0.012	1.42 ± 0.595
C = *Azotobacter* sp. K4 1.0%	0.017 ± 0.009	0.072 ± 0.015	1.07 ± 0.774
D = *Azotobacter* sp. S2 0.5%	0.020 ± 0.013	0.081 ± 0.030	1.20 ± 0.712
E = *Azotobacter* sp. S2 1.0%	0.019 ± 0.009	0.065 ± 0.026	1.40 ± 0.450
F = *Azotobacter* sp. K4+S2 0.5% respectively	0.017 ± 0.009	0.078 ± 0.052	1.68 ± 1.086
G = *Azotobacter*sp. K4+S2 1.0% respectively	0.022 ± 0.010	0.090 ± 0.059	1.27 ± 0.380

Description: Mean ± standard deviation value

Population of *Azotobacter* sp.

*Azotobacter*sp. inoculation either single or mixed isolate on its population in the rhizosphere increased its population in rhizosphere (Table 2).Heigher population was in rhizosphere of soybean inoculated with 1.0% of mixed isolate of*Azotobacter* sp. K4+S2 1.0%. The lowest one was observed in control treatment. Inoculation of 10% of *Azotobacter* sp. K4+S2 increased population of *Azotobacter*up to73.33% compared to control. Liquid inoculants of *Azotobacter* sp. K4 and S2 isolates (Figure 1) can proliferated in rhizosphere since both isolate was isolated from saline soils. Thus the bacteria would bemore easy to adapt. Kizilkaya (2009) reported that indigenous*Azotobacter*isolates were more adaptive and competitive compared to non-indigenous when inoculated in soil.

*Azotobacter*sp. has the ability to synthesize proline and glycine betaine as osmoprotectant agents in response to osmotic stress in saline condition (Naz *et al.*, 2012; Omer *et al.*, 2016).*Azotobacter* sp. could stand for salt tolerance up to 10% NaCl (Akhter *et al.*, 2012 and Dhanraj *et al.*, 2012), while saline soils contain about 2 to 6% NaCl (Djukri, 2009), thus *Azotobacter* sp. could live in saline soils that used in this experiment.



(a) (b)

Figure 1. Light microscopy images of *Azotobacter* sp. K4 isolate (a) and S2 isolate (b)

Table 2. Populationof *Azotobacter* sp. in soybean rhizosphere 37 days afterinoculation of *Azotobacter* sp

Treatments	Population of *Azotobacter* sp. (CFU g^{-1})
A = Without inoculation	$4.0 \times 10^4 \pm 1.4 \times 10^4$
B = *Azotobacter* sp. K4 0.5%	$1.1 \times 10^5 \pm 2.3 \times 10^4$
C = *Azotobacter* sp. K4 1.0%	$7.8 \times 10^4 \pm 4.8 \times 10^4$
D = *Azotobacter* sp. S2 0.5%	$5.1 \times 10^4 \pm 2.8 \times 10^4$
E = *Azotobacter* sp. S2 1.0%	$6.7 \times 10^4 \pm 1.7 \times 10^4$
F = *Azotobacter* sp. K4+S2 0.5% respectively	$1.2 \times 10^5 \pm 2.5 \times 10^4$
G = *Azotobacter*sp. K4+S2 1.0% respectively	$1.5 \times 10^5 \pm 1.0 \times 10^5$

Description: Mean ± standard deviation values

Plant Height and Number of Leaves of Soybean

Growth of soybean was retarded compared to normal plant grown in non-saline soil. Indeed, clayed soil in this experiment limited root growth so that soybean shoot and height were smaller than we expect (Figure 2).The height of soybean on saline soils tended to be lower than in non-saline soils described by Triyani *et al.*(2013). Plant height of all plant inoculated with *Azotobacter*sp. was higherthan that of control (Table 3). Higher plant height was recorded in soybean received 1.0% of *Azotobacter* S2 although the standard deviation was high. The high salt and clay particles on saline soils (79%) cause low aeration,percolation, and porosity (Szombathova *et al.*, 2008). Saline soils with a high clay particles have a lot of micropores due to dense structure, thus it generates the little space between the soil aggregates (Hanafiah, 2005).This condition caused soybean difficult to absorb nutrients and decreased the plant height.

Table 3. The effect of *Azotobacter* sp. inoculation on plant height of soybean at 28 and 35 DAS

Treatments	Plant height (cm)	
	28 DAS	35 DAS
A = Without inoculation	19.18 ± 6.28	20.68 ± 6.54
B = *Azotobacter* sp. K4 0.5%	23.18 ± 3.14	23.67 ± 2.51
C = *Azotobacter* sp. K4 1.0%	22.03 ± 4.34	23.64 ± 5.60
D = *Azotobacter* sp. S2 0.5%	24.78 ± 5.83	26.80 ± 6.35
E = *Azotobacter* sp. S2 1.0%	30.40 ± 16.57	33.20 ± 17.49
F = *Azotobacter* sp. K4+S2 0.5% respectively	25.23 ± 5.30	28.85 ± 10.75
G = *Azotobacter*sp. K4+S2 1.0% respectively	20.00 ± 2.35	21.20 ± 3.01

Description: Mean ± standard deviation values

Figure 2 showed that the number of leaves of soybean at 28 and 35 DAS relatively uniform in each treatment; between 2.00-3.25 due their genetic characteristic. However,

inoculation of *Azotobacter* sp. S2 1.0% increased plant height up to 36.91% compared to the control. This indicates that *Azotobacter* sp. has the potential to increase vegetative growth of soybean plants through the production of phytohormones auxin and cytokinin to induce cell division (Hindersah and Simarmata, 2004).

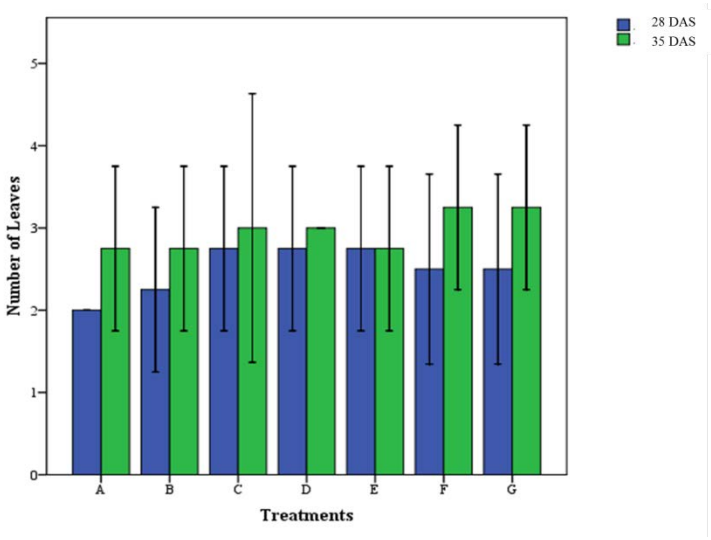


Figure 2. The effect of *Azotobacter* sp. inoculation on number of leaves of soybean at 28 and 35 DAS. Vertical bar indicated standard deviation.

Shoot-Root Ratio of Soybean

Inoculation of*Azotobacter* sp. increased shoot-root ratio (S/R) which was indicated the shoot growth was superior than root growth (Figure 3).Inoculation of 0.5% of *Azotobacter*sp. isolates K4+S2gave the highest S/R (Table 4). This treatment increased shoot-root ratio up to42.74% over control. Data in Table 4 also indicated thatroot growthwas disturbed (Yoon *et al.*, 2009), so the photosynthate flow was used for shoot growth. The decrease in root growth caused by the competition between Na^+ and K^+ on saline soils which inhibited the absorption of K^+ and disturbed the nutrient balance, osmotic regulation, and caused spesific ion toxicity (Nadeem *et al.*, 2014). This study indicated that higher shoot length could be caused by cytokines production by *Azotobacter* sp. that was adsorbed by root and transported by the xylem to the target cells in the shoot (Kudo *et al.*, 2010).

Table4. The effect of *Azotobacter* sp. inoculation ondry weight of shoot, root, and shoot-root ratio of soybean at 37 DAS

Treatments	Shoot-Root Ratio (g)
A = Without inoculation	0.43 ± 1.19
B = *Azotobacter* sp. K4 0.5%	0.64 ± 2.14
C = *Azotobacter* sp. K4 1.0%	0.51 ± 1.23
D = *Azotobacter* sp. S2 0.5%	0.60 ± 0.29
E = *Azotobacter* sp. S2 1.0%	0.71 ± 2.12
F = *Azotobacter* sp. K4+S2 0.5% respectively	0.74 ± 2.08
G = *Azotobacter*sp. K4+S2 1.0% respectively	0.57 ± 0.52

Description: Mean ± standard deviation values

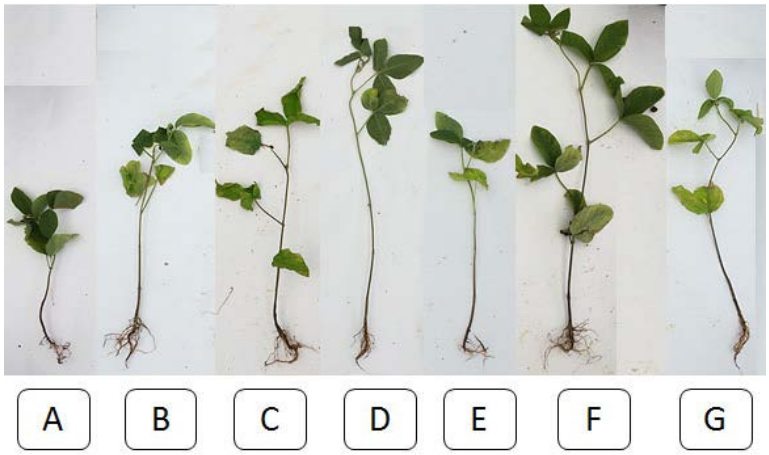


Figure 3. Soybean plant performance at 37 days after sowing in all *Azotobacter*sp. treatment and control one(A: control, B:K4 0.5%, C:K4 1.0%, D: S2 0.5%, E: S2 1.0%, F: K4+S2 0.5%, and G: K4+S2 1.0%)

CONCLUSIONS

The results of this study clearly revealed that inoculation of *Azotobacter* sp. liquid culturepotentially increased available N, N uptake, population of *Azotobacter* sp., plant height, number of leaves and dry weight of shoot and root of soybean var. Groboganon saline soils compared to that of control; inoculation of *Azotobacter* sp. S2 1.0% increased plant height up to 36.91%; inoculation of *Azotobacter* sp.K4+S2 0.5% respectively increased N uptake and shoot-root ratio by 60.12% and 42.74% respectively compared to control; The experiment showed that the available $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$,and population of *Azotobacter*increased up to 45.45%, 37.78%, and 73.33% after 1,0% of mixed *Azotobacter*. This research suggested that nitrogen availability insaline soils could be enhanced by saline tolerant *Azotobacter* sp.; which is in turn increased soybean growth.

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